

# RESEARCH LETTER

## LysK CHAP endopeptidase domain is required for lysis of live staphylococcal cells

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### Keywords

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### Abstract

LysK is a staphylococcal bacteriophage endolysin composed of three domains: an N-terminal cysteine, histidine-dependent amidohydrolases/peptidases (CHAP) endopeptidase domain, a midprotein amidase 2 domain, and a C-terminal SH3b\_5 (SH3b) cell wall-binding domain. Both catalytic domains are active on purified peptidoglycan by positive-ion electrospray ionization MS. The cut sites are identical to LytA (phi11 endolysin), with cleavage between D-alanine of the stem peptide and glycine of the cross-bridge peptide, and N-acetylmuramoyl-L-alanine amidase activity. Truncations of the LysK containing just the CHAP domain lyse *Staphylococcus aureus* cells in zymogram analysis, plate lysis, and turbidity reduction assays but have no detectable activity in a minimal inhibitory concentration (MIC) assay. In contrast, truncations harboring just the amidase lytic domain show faint activity in both the zymogram and turbidity reduction assays, but no detectable activity in either plate lysis or MIC assays. A fusion of the CHAP domain to the SH3b domain has near full-length LysK lytic activity, suggesting the need for a C-terminal binding domain. Both LysK and the CHAP-SH3b fusion were shown to lyse untreated *S. aureus* and the coagulase-negative strains. In the checkerboard assay, the CHAP-SH3b fusion achieves the same level of antimicrobial synergy with lysostaphin as the full-length LysK.

### Introduction

Staphylococci are Gram-positive pathogens that negatively impact both human and livestock health. Recent clinical estimates indicate that in 2005, multiple drug resistant forms of *Staphylococcus aureus* (methicillin-resistant *S. aureus*; MRSA) resulted in > 94 000 serious infections and > 18 000 deaths in the United States, a larger number than associated with deaths due to AIDS (Klevens *et al.*, 2007). Multiple drug resistance is not unique to human pathogens. MRSA is also appearing in livestock outside the United States (bovine, porcine, and poultry) (Lee, 2003; Voss *et al.*, 2005; Nunan & Price, 2007) as well as companion animals (dogs, cats, and horses) (Nunan & Price, 2007; Leonard & Markey, 2008). Regardless of antibiotic resistance phenotype, staphylococci are a persistent problem in the dairy industry resulting in an estimated loss of 2 billion

dollars annually to US dairy producers (Sordillo & Streicher, 2002). In one animal study, *S. aureus* and coagulase-negative staphylococci (CoNS) were leading causes of bovine mastitis, accounting for > 40% of all cases (Wilson *et al.*, 1997). Conventional mastitis treatment (antibiotics), has been shown to be only marginally effective (Deluyker *et al.*, 2005). Identifying novel antimicrobials to treat staphylococcal infections in both humans and animals, particularly antimicrobials that avoid resistance development, is essential to address this global problem.

Bacteriophage endolysins are a potential source of narrow-spectrum antimicrobials for treatment of antibiotic-resistant pathogens. Phage endolysins are normally produced at the end of the bacteriophage lytic cycle to degrade the host cell wall peptidoglycan, allowing turgor pressure to rupture the cell membrane and release nascent viral progeny (Loessner, 2005). When exposed exogenously

to the target pathogen, endolysins can degrade the Gram-positive peptidoglycan and cause exolysis (or 'lysis from without'). The peptidoglycan structure is highly conserved within any given genus, but can have species-specific changes (Schleifer & Kandler, 1972). As a result, peptidoglycan hydrolases are often genus specific, if not species specific (Loessner, 2005; Borysowski *et al.*, 2006). Because of the near species specificity of these peptidoglycan hydrolases, phage endolysins represent a novel class of antimicrobials that might be useful in avoiding broad-range antibiotic use, often associated with resistance development (Spratt, 1994). Phage lysins have the further advantage that they may be refractory to resistance development, as no bacterial strains resistant to their phage endolysins have been identified despite repeated attempts to find them (Loeffler *et al.*, 2001; reviewed in Fischetti, 2005).

Previous work has shown that LysK, the recombinant endolysin from the staphylococcal phage K (O'Flaherty *et al.*, 2004), when added exogenously, is able to lyse agriculturally relevant staphylococci as well as clinically relevant MRSA (O'Flaherty *et al.*, 2005). LysK has also been shown to be synergistic with the bacteriocin lysostaphin to inhibit *S. aureus* growth (Becker *et al.*, 2008). In this study, LysK cut sites in *S. aureus* peptidoglycan were determined via positive-ion electrospray ionization MS (EIMS) of LysK digestion products. In addition, three peptidoglycan hydrolase assays have been utilized to quantify the lytic activity levels of LysK and selected deletion constructs on live cells.

LysK deletion constructs were created to systematically isolate the lytic domains of LysK to determine the contribution of each to exolysis. Finally, the lytic activity of LysK and a high-activity deletion construct were compared to determine their ability to lyse multiple species of CoNS.

## Materials and methods

### Plasmids, constructs, and strains

The lysK cDNA was kindly provided by Paul Ross (O'Flaherty *et al.*, 2005). Phage K genomic sequence has been published (AY176327) and the LysK protein sequence is also available (AA047477.2) through GenBank. The parental vector pSB0001 is the wild-type lysK cDNA subcloned between the NdeI and XhoI sites in the inducible expression vector pET21a (EMD Biosciences, San Diego, CA) described previously as LysK-pET21a (Becker *et al.*, 2008). Plasmids and primers used in this study are described in Table 1.

Truncations of the lysK gene were produced using standard PCR cloning methods with the lysK plasmid pSB0001 as template and engineered restriction sites introduced via primer design (as described previously) (Donovan *et al.*, 2006b). Plasmid pSB0801 was generated by digesting the PCR product generated with template pSB0001 and primers LysKSH3bSalI and pET21aStyIR with the restriction enzymes SalI and StyI and ligating the resulting DNA fragment into the XhoI- and StyI-digested pSB0201. This ligation

**Table 1.** Plasmids and primers

Plasmids	Proteins produced	Forward primers	Reverse primers	Recipient vectors
pSB0001	LysK			
pSB0201	LysK221	pET21aXbaI-F	lysKCHAP221XhoI-R	pET21a
pSB1201	LysK297	pET21aBglII-F	lysK300XhoI-R	pET21a
pSB0301	LysK390	pET21aXbaI-F	lysKCA390XhoI-R	pET21a
pSB0701	LysK149-495	lysKAmid149XbaI-F	lysKXhoI-R	pET21a
pSB0105	LysK325-495	lysKAmid149XbaI-F*	lysKXhoI-R*	pET21a
pSB0801	LysKΔ221-390	lysKSH3bSalI-F	pET21aStyI-R	pSB0201
Primers	Sequences			
lysKXhoI-R	5'-ATGGTGATG <u>CTCGAG</u> TTTGAATACTCC			
pET21aXbaI-F	5'-GGATAACAATTCCCC <u>TCTAG(a)</u>			
lysKCHAP221XhoI-R	5'-GTATTG <u>CTCGAGT</u> GAAGAAGACCTGC			
pET21aBglII-F	5'-CGTAGAGGATCG <u>AGATCT</u> CGATC			
lysK300XhoI-R	5'-GAATACTGC <u>CTCGAG</u> ATTTTAAAGG			
lysKCA390XhoI-R	5'-GTGGTG <u>CTCGAG</u> ACTGCGCTACTTGTTTACC			
lysKAmid149XbaI-F	5'-TCCCC <u>TCTAGA</u> AATAATTTTGTTAACTTTAAGAA GGAGATATACATATGGTAGATAATTATTACGG			
lysKSH3bSalI-F	5'-CAAGT <u>GTCGAC</u> AGTACACCGGC			
pET21aStyI-R	5'-CGTTTAGAGGCC <u>CCAAGG</u> GTTATG			

\*Digestion with restriction enzymes NdeI and XhoI. Underlined and bold sequences indicate the restriction enzyme sites introduced and enzymes used with all nonasterisked engineered primers.

introduces two amino acids (LD) at the fusion joint, destroying the original vector XhoI site. All pET21a-derived constructs have an additional eight amino acids at the C-terminus composed of LE introduced as an XhoI restriction site, and a 6xHis tag for nickel chromatography purification. All subcloning was performed in *Escherichia coli* DH5 $\alpha$  (Invitrogen, Carlsbad, CA) for plasmid DNA isolation and sequence verification. pET21a constructs were expressed in *E. coli* BL21 (DE3) (EMD Biosciences).

Staphylococci were grown at 37 °C in either brain–heart infusion broth (BHI) (BD, Sparks, MD) for turbidity-reduction assays and zymograms or tryptic soy broth (TSB) (BD) for minimal inhibitory concentration (MIC) and plate lysis assays. All CoNS strains are mastitis isolates and gifts from Max Paape, except for *Staphylococcus hyicus*, a gift from David Kerr (University of Vermont). The *S. aureus* strain Newman was a gift from Jean Lee, Channing Lab, Brigham Young Women's Hospital, and Newbould 305 was purchased from American Type Culture Collection (ATCC) (29740). The identity of each strain was verified by the Animal Health Diagnostic Lab, Maryland Department of Agriculture, College Park, MD. All assays were performed with *S. aureus* strain Newman unless otherwise noted.

### Protein purification

LysK truncated protein constructs were expressed and purified by Ni-NTA chromatography (Qiagen) as per the manufacturer's instructions, with the following modifications. After coincubation of His-tagged proteins and Ni-NTA, bound Ni-NTA resin was pelleted by centrifugation at 200 g for 10 min, the supernatant was removed, and the resin was resuspended in 5 mL wash buffer (50 mM NaH<sub>2</sub>PO<sub>4</sub>, 300 mM NaCl, pH 8.0) with 10 mM imidazole. The wash and elution buffer profiles were empirically determined to be 20 mL of wash buffer with 10 mM imidazole, 10 mL with 20 mM imidazole, 10 mL with 60 mM imidazole, and elution with 1.8 mL wash buffer with 250 mM imidazole. Immediately after purification from the nickel column, all samples were made up to 1% glycerol to reduce precipitation of the purified protein. All samples were then converted to a low-salt buffer (150 mM NaCl, 10 mM Tris-HCl, 1% glycerol, pH 7.5) via Zeba desalting column (Pierce) and 0.22  $\mu$  filter sterilized. Protein concentration determinations were made via BCA Protein kit (Pierce). Sterilized protein preparations were stored at 4 °C until the time of the assay.

### LysK digestion of *S. aureus* cell wall preparations

Digestions were initiated by the addition of 20  $\mu$ L of 2 mg mL<sup>-1</sup> LysK in buffer (400 mM NaCl, 20 mM Tris-HCl, 1% glycerol, 10 mM dithiothreitol) to 980  $\mu$ L of a *S. aureus*

cell wall suspension (OD<sub>600 nm</sub> = 1.0) in 25 mM Tris-HCl, 400 mM NaCl, pH 7.4. Cell walls were prepared as described previously (Pritchard *et al.*, 2004). After digestion at 37 °C for 2 h, the reaction mixture was clarified by centrifugation and the supernatant was ultrafiltered using a 5000-MW cutoff Vivaspin 500 (Sartorius North America Inc., NY). The filtrate was then applied to a Sep-Pak C18 cartridge (Water Corporation, Milford, MA) that had been activated by washing with 2 mL methanol and then rinsed with water. Unbound material was washed through the column with 5 mL water and bound peptide was eluted with 3 mL of 50% (v/v) methanol in water. The eluted sample was dried in a SpeedVac and redissolved in 100  $\mu$ L water. A 1- $\mu$ L aliquot was analyzed by positive-ion EIMS performed on a Micro-mass Q-TOF2 mass spectrometer (Micromass Ltd, Manchester, UK).

### Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and zymogram

The purified LysK protein, deletion constructs and Kaleidoscope protein standards (Bio-Rad) were analyzed with 15% SDS-PAGE, with or without a 300 mL equivalent of midlog phase (OD<sub>600 nm</sub> = 0.4–0.6) *S. aureus* strain Newman cells embedded in the gel (zymogram). LysK protein constructs were boiled in Laemmli sample buffer (Bio-Rad) with  $\beta$ -mercaptoethanol, and electrophoresed simultaneously (identical conditions and times) in both the SDS-PAGE and the zymogram using the BioRad Mini-PROTEAN 3 dual gel apparatus, according to the manufacturer's instructions. SDS gels were Coomassie stained and zymograms were washed in excess water for 30 min and incubated at room temperature in water for up to 48 h. Areas of clearing in the turbid gel indicate a lytic protein in the gel.

### Plate lysis assay

Purified proteins for each construct were diluted in saline Tris lysis buffer (STB; 150 mM NaCl, 10 mM Tris-HCl, pH 7.5), and 10  $\mu$ L of 10-fold serial dilutions 1  $\mu$ g  $\mu$ L<sup>-1</sup> (10  $\mu$ g), 0.1  $\mu$ g  $\mu$ L<sup>-1</sup> (1  $\mu$ g), and 0.01  $\mu$ g  $\mu$ L<sup>-1</sup> (0.1  $\mu$ g) were spotted onto a freshly spread lawn of log phase cells that had air dried for 30 min on tryptic soy agar plates. The spotted plates were air dried for 10 min in a laminar flow hood, and incubated overnight at 37 °C. Cleared spots were digitally photographed within 20 h of plating the cells.

### Turbidity reduction assays

Freshly grown cell substrates for the turbidity reduction assay were prepared from midlog phase cultures (OD<sub>600 nm</sub> = 0.4–0.6) grown in BHI at 37 °C, harvested by centrifugation at 4 °C, and stored on ice. Immediately before the assay, cells were resuspended in STB such that when

added to 100  $\mu$ L of lysis buffer  $\pm$  protein, the final solution has an OD<sub>600 nm</sub> of *c.* 1.0. Alternatively, for ease of assay preparation, and to reduce the variability of the assay (S. Becker, unpublished data), frozen cell substrate was prepared in large cultures of *S. aureus* strain Newman, harvested as above, pooled, resuspended in STB, supplemented with 25% glycerol, and frozen at  $-80^{\circ}\text{C}$ . As needed for turbidity assays, the frozen cells were thawed, washed three times with STB, and resuspended as with fresh cells. Although previously frozen, these cells are cryopreserved and will grow in culture after freezing.

The assays were performed in a Molecular Devices, Spectra Max 340 plate reader as described previously (Becker *et al.*, 2008). All samples were performed in triplicate wells with a parallel triplicate 'no enzyme' buffer control. OD<sub>600 nm</sub> readings are taken automatically every 20 s for the duration of the assay. The readings for each well were analyzed in a sliding 40 s window over each group of three consecutive time points to identify the highest instantaneous change in OD<sub>600 nm</sub> for each well. The highest  $\Delta\text{OD}_{600\text{ nm}}$  value for each triplicate experimental sample was averaged and the highest cell-alone control value (calculated in the same manner) was then subtracted. The averaged values are multiplied by 1.5 to give a  $\Delta\text{OD}_{600\text{ nm}}\text{ min}^{-1}$ , then divided by micrograms of enzyme protein in the sample to yield a specific activity ( $\Delta\text{OD}_{600\text{ nm}}\mu\text{g}^{-1}\text{ min}^{-1}$ ).

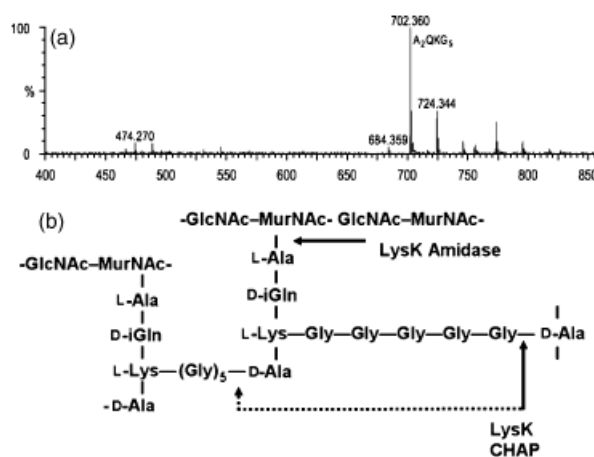
### MIC and checkerboard assays

A microdilution broth method for determination of the MIC was used (Jones *et al.*, 1985), with these modifications. All MICs were performed in TSB corrected for STB addition (50% lysis buffer  $\pm$  protein and 50%  $2\times$  TSB). Modified checkerboard assays were performed as described previously, reducing the range of the assay to three 10-fold serial dilutions (Becker *et al.*, 2008) to determine synergy between lysostaphin and the high-activity LysK constructs. Each MIC assay and checkerboard assay represents at least two determinations per experiment.

## Results

### The enzymatic domains of LysK are both active on purified peptidoglycan

To determine the LysK peptidoglycan cut sites, EIMS was performed on LysK-digested *S. aureus* cell wall preparations. The primary product of LysK digestion was A<sub>2</sub>QKG<sub>5</sub>, which in EIMS yields a peak with  $m/z=702$  (Fig. 1a). To generate this fragment, two enzymatic activities must be present, an amidase that cleaves between the *N*-acetylmuramic acid and *L*-alanine of the stem peptide, and an endopeptidase that cleaves at the termini of the crossbridge, or at a consistent site within the pentaglycine crossbridge. MS/MS of the  $m/z=702$  ion gave a prominent peak at  $m/z=286$  due to a

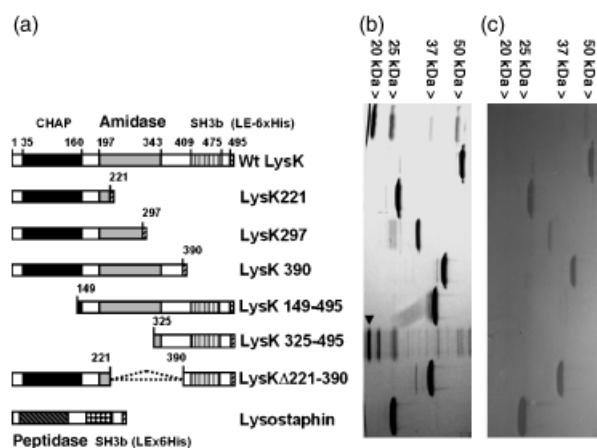


**Fig. 1.** EIMS spectrum of purified *Staphylococcus aureus* peptidoglycan products of LysK digestion. (a). The LysK cleavage sites that result in the branched peptide A<sub>2</sub>QKG<sub>5</sub> of  $m/z$  702 are indicated (b). The peak at  $m/z$  724 is believed to be the sodium adduct of the  $m/z$  702 peak.

pentaglycine fragment, indicating that the endopeptidase cleavage occurred between the *D*-alanine and the first glycine in the cross-bridge (Fig. 1b). Other ions in the MS/MS spectrum including  $m/z=432$  (KG<sub>5</sub>) and  $m/z=503$  (AKG<sub>5</sub>) are also consistent with this interpretation. Additionally, codigestion with both the enzymatic activities of LysK and the known enzymatic activity of lysostaphin generated EIMS peaks at 702 (A<sub>2</sub>QKG<sub>5</sub>), 645 (A<sub>2</sub>QKG<sub>4</sub>), 588 (A<sub>2</sub>QKG<sub>3</sub>), and 531 (A<sub>2</sub>QKG<sub>2</sub>), confirming this interpretation (data not shown). Identical cleavage sites have been reported for the *c.* 40% identical phi11 endolysin LytA (Navarre *et al.*, 1999).

### The LysK CHAP domain is sufficient for exolysis of *S. aureus* cells

LysK contains three domains: an N-terminal cysteine, histidine-dependent amidohydrolase/peptidase (CHAP) domain, a midprotein amidase-2 domain, and a C-terminal SH3b cell wall-binding domain (O'Flaherty *et al.*, 2005). To determine if all three domains are necessary for exolysis, five truncation or deletion constructs were generated to isolate each domain on a separate construct or in pairwise combinations with the other domains. Schematics of each construct are presented in Fig. 2a. Lysostaphin, a potent anti-staphylococcal bacteriocin, glycyl-glycine endopeptidase peptidoglycan hydrolase, is included as a positive control and is composed of an N-terminal endopeptidase domain and a C-terminal SH3b domain. Eight micrograms of each protein was analyzed in both a 15% SDS-PAGE and an identical zymogram (Fig. 2c and b). All construct proteins are of the expected molecular weight and  $>95\%$  pure, with the exception of the SH3b domain alone (LysK325-495) that has multiple contaminating bands.



**Fig. 2.** Schematic representation, SDS-PAGE and zymogram analysis of lysostaphin and LysK deletion constructs. (a) Schematic of LysK protein constructs. Wt LysK: CHAP endopeptidase (black), amidase domain (gray), SH3b domain (vertical stripes); lysostaphin: glycyl-glycine endopeptidase domain (diagonal stripes), SH3b domain (black and white checkerboard). (b) SDS-PAGE: 4  $\mu$ g of each construct was loaded per lane. The carrot (▶) indicates the band for the LysK 325–495 truncation, per calculated molecular weight. (c) Zymogram: 4  $\mu$ g of each construct and excess prestained Kaleidoscope protein standards (Bio-Rad), to allow visualization of the molecular weight markers, were added to the zymogram. The predicted molecular weights for each construct protein are Wt LysK, 55.8 kDa; LysK221, 25.7 kDa; LysK297, 33.9 kDa; LysK390, 44.4 kDa; LysK149–495, 39.3 kDa; LysK 325–495, 19.7 kDa; LysK221–390, 37.4 kDa; and lysostaphin, 27.9 kDa.

Zones of clearing in the zymogram correspond to the predicted molecular weights and positions of the purified proteins in the SDS-PAGE (Fig. 2c). All constructs containing a CHAP domain cleared the zymogram within 30 min after washing. As expected, the LysK325–495 protein, containing only the SH3b domain, did not show any detectable clearing in the zymogram. Additional incubation times showed slight clearing for the LysK149–495 construct, harboring just the amidase and SH3b domains. However, the clearing was barely visible to the naked eye, even after 48 h, and is not readily discernable in Fig. 2. LysK297, harboring the intact CHAP domain and part of the amidase domain, does not show as robust clearing in the zymogram as the LysK 221, harboring just the CHAP domain. Lysostaphin shows a cleared zone of similar intensity as the wild-type LysK, LysK221, LysK390, and LysKΔ221–390.

### The LysK SH3b domain increases the CHAP domain activity in plate lysis, turbidity reduction, and MIC assays

LysK deletion constructs were tested in three antimicrobial assays: plate lysis, turbidity reduction, and MIC. Constructs lacking the SH3b domain (LysK211 and LysK390), show at least a 10-fold reduced activity compared with wild-type

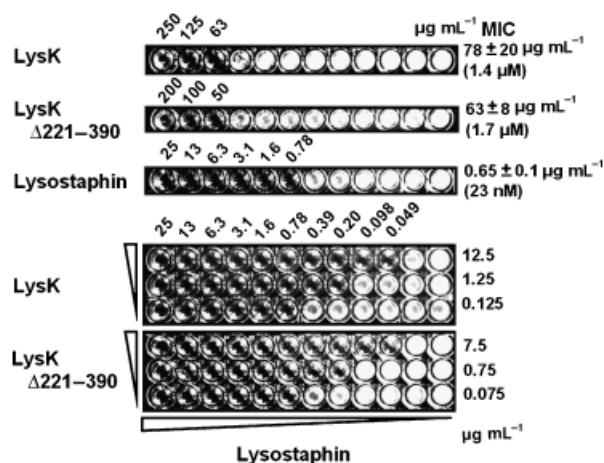
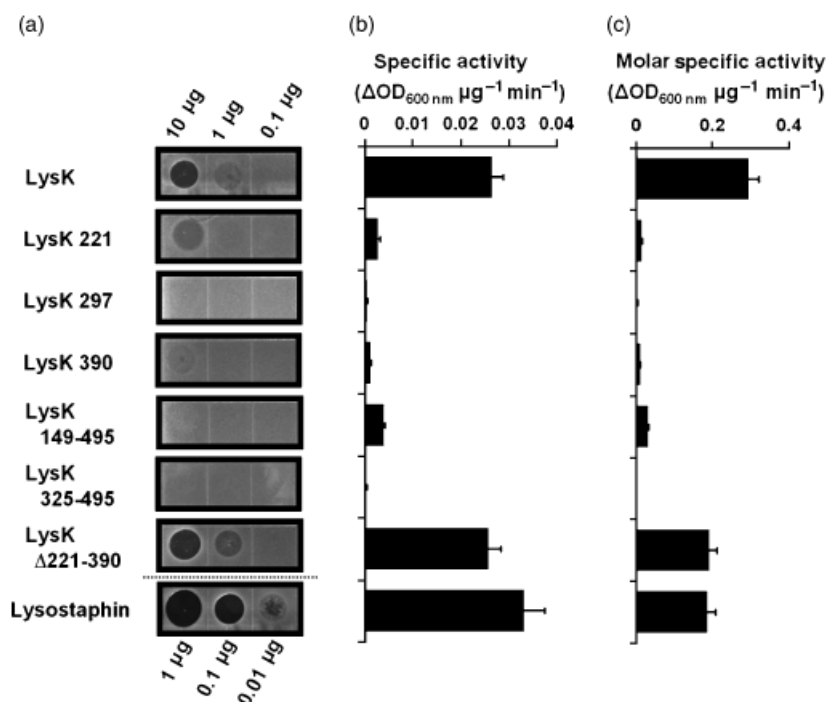
LysK. The SH3b domain alone, LysK325–495, again had no discernable activity on the plate lysis assay. In contrast to the zymogram, 10  $\mu$ L spots containing either 10  $\mu$ g of LysK297 (29  $\mu$ M) or LysK149–495 (25  $\mu$ M) had no discernable activity in the plate lysis assay (Fig. 3a). Fusion of the SH3b domain to the CHAP domain (LysK221–390) increased the activity of the CHAP domain (1  $\mu$ g/39  $\mu$ M–0.1  $\mu$ g/1.8  $\mu$ M) to approximately the wild type LysK levels (0.1  $\mu$ g/2.7  $\mu$ M). Lysostaphin showed demonstrable clearing at a lower dosage, lysing when 0.01  $\mu$ g was spotted in 10  $\mu$ L (36 nM).

Turbidity reduction assays with each deletion construct were performed on frozen *S. aureus* strain Newman cells. The turbidity reduction assays were performed with physiological saline (0.15 M NaCl) to better approximate those conditions where an antimicrobial might be used. LysK, LysK221–390 and lysostaphin (high-activity proteins) were assayed with 5  $\mu$ g of lysin (final concentrations of 450, 670, and 900 nM, respectively) to remain in the linear range of LysK in the assay (Becker *et al.*, 2008). Because of the weak activity (near background) for the remaining constructs, 10  $\mu$ g ( $2 \times$ ), of each construct, was used to generate a measureable signal. For these constructs, LysK221, LysK297, LysK 390, and LysK149–495, their final concentrations were 1.9, 1.5, 1.1, and 1.3  $\mu$ M. When compared on a mass level, the specific activity of wild-type LysK and the amidase deletion construct, LysK221–390, are nearly identical (Fig. 3b). When corrected for molarity, the mean difference was also found not to be statistically significant (*t*-test for independent samples, two tailed,  $\alpha = 0.05$ ; d.f. = 6), although it appears to be greater (Fig. 3c).

Although it is known that different assays for peptidoglycan hydrolase activity do not always agree quantitatively (Kusuma & Kokai-Kun, 2005), the turbidity reduction and plate lysis assay results are very consistent. Deletion of the SH3b domain yields at least a 10-fold reduction in specific activity for all lytic domains in both assays. Deletion of the amidase domain appears to have no significant effect (LysK221–390 vs. LysK) when compared on either a mass or molar ratio (*t*-test for independent samples, two tailed, d.f. = 6,  $\alpha = 0.05$ ), and might even slightly enhance the activity of some constructs in these lytic assays (LysK221 relative to LysK390). As in the plate lysis assay, LysK 297 and LysK 325–495 show no activity in turbidity reduction assays. Unlike the plate lysis assay, the amidase+SH3b domain construct (LysK149–495) showed significantly greater activity relative to the other low-activity truncations, suggesting that while the amidase domain is insufficient to prevent plate growth, it has some activity on *S. aureus* cells in solution.

The MIC of each protein was determined in TSB. As in both the plate lysis assays and the turbidity-reduction assays, deletion of the SH3b domain significantly reduces the activity of the LysK truncations. LysK221, LysK297,

**Fig. 3.** Comparison of LysK deletion construct antimicrobial activity in plate lysis and turbidity reduction assays. (a) Representative plate lysis assay with *Staphylococcus aureus* strain Newman on tryptic soy agar plates, incubated overnight at 37 °C. The lowest concentration at which cleared zones were detected for each 10- $\mu$ L spot of protein are Wt LysK, 1  $\mu$ g (1.8  $\mu$ M); LysK  $\Delta$ 221, 10  $\mu$ g (39  $\mu$ M); LysK390, 10  $\mu$ g (23  $\mu$ M); LysK $\Delta$ 221-390, 1  $\mu$ g (2.7  $\mu$ M); and lysostaphin, 0.1  $\mu$ g (36 nM). (b) Turbidity reduction assay specific activities for each protein are reported as a maximum rate derived from a 5-min assay previously frozen *S. aureus* strain Newman cells. (c) Turbidity reduction assay specific activities when corrected for molarity. Error bars represent the SE of the mean for triplicate samples in four independent experiments.



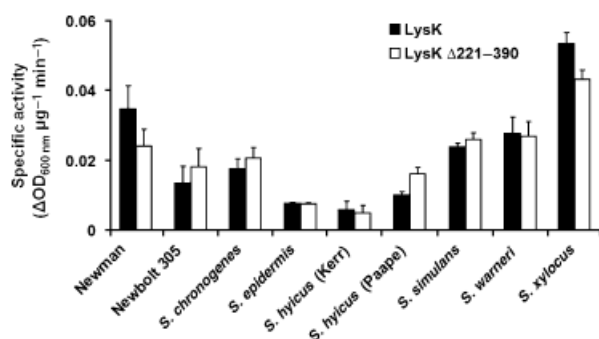
**Fig. 4.** MIC of purified LysK and LysK $\Delta$ 221-390 (above) and synergistic interaction with lysostaphin (below). Error indications represent the SE of the mean.

LysK390, and LysK149-495 were unable to inhibit cell growth when used at the maximum concentrations achievable [600  $\mu$ g mL<sup>-1</sup> (23  $\mu$ M), 180  $\mu$ g mL<sup>-1</sup> (5  $\mu$ M), 650  $\mu$ g mL<sup>-1</sup> (15  $\mu$ M), and 1.2 mg mL<sup>-1</sup> (31  $\mu$ M), respectively]. Proteins that could inhibit cell growth in the MIC assay at concentrations < 100  $\mu$ g mL<sup>-1</sup> were studied further (Fig. 4). Both the wild-type LysK protein and the amidase-deleted protein, LysK $\Delta$ 221-390, have similar MICs (78  $\pm$  20 and 63  $\pm$  8  $\mu$ g mL<sup>-1</sup>, and MIC molar concentrations 1.4  $\pm$  0.34 and 1.8  $\pm$  0.23  $\mu$ M, respec-

tively), indicating that the presence of the amidase domain is not required for wild-type levels of growth inhibition. When tested in the checkerboard assay (Fig. 4), the internal amidase deletion, LysK $\Delta$ 221-390, shows the same level of synergistic antimicrobial activity with lysostaphin as does the full-length LysK. LysK enhanced the activity of lysostaphin 16-fold at 12.5  $\mu$ g mL<sup>-1</sup> (220 nM) and fourfold at 1.25  $\mu$ g mL<sup>-1</sup> (22 nM). The amidase-deletion enhanced the activity of lysostaphin 16-fold at 7.5  $\mu$ g mL<sup>-1</sup> (220 nM) and fourfold at 0.75  $\mu$ g mL<sup>-1</sup> (20 nM) (Fig. 4; Becker *et al.*, 2008). These results again suggest the CHAP domain accounts for nearly all of the antimicrobial properties of LysK. The fractional inhibitory concentration (FIC) determination between LysK and lysostaphin is highly significant with FIC < 0.5 (Becker *et al.*, 2008).

### CoN are sensitive to the exolytic activity of LysK and LysK $\Delta$ 221-390

LysK and the highly active deletion construct (LysK $\Delta$ 221-390) were tested in turbidity-reduction assays against multiple live staphylococcal strains (Fig. 5). The strains tested include the human pathogen *S. aureus* strain Newman, the mastitis *S. aureus* strain Newbould 305, and the CoNS mastitis-causing strains *Staphylococcus chronogenes*, *Staphylococcus epidermis*, *S. hyicus*, *Staphylococcus simulans*, *Staphylococcus warneri* and *Staphylococcus xylocus*. *Staphylococcus hyicus* (Paape) and *S. xylocus* were the only strains



**Fig. 5.** Staphylococcal species specificity of LysK deletion constructs in turbidity-reduction assays. Five micrograms of LysK (black bars; final concentration 0.45 μM) or LysKΔ221-390 (white bars; final concentration 0.67 μM) were exposed to midlog phase staphylococcal cells resuspended to an OD<sub>600 nm</sub> in a volume of 200 μL. Maximum rate of turbidity reduction over the 5-min assay are reported as specific activity with error bars representing the SE of the mean of four independent experiments with triplicate samples.

with significantly different mean values between treatments when analyzed by ANOVA using the *t*-test, for independent samples for *post hoc* analysis (two tailed, d.f. = 6,  $\alpha$  = 0.05).

## Discussion

The LysK peptidoglycan cut sites have been determined and are identical to the phi11 endolysin cut sites (Navarre *et al.*, 1999). Analysis of LysK deletion construct activity measurements indicate that the CHAP domain plays a crucial role in exolysis, with the amidase domain contributing very little, if any, lytic activity. The activity of the CHAP domain is greatly enhanced, to near wild-type levels, by the presence of the LysK SH3b domain. This CHAP domain lytic activity is synergistic with lysostaphin, consistent with previous findings with LysK (Becker *et al.*, 2008). The LysK-susceptibility profile of staphylococcal strains obtained via turbidity reduction assays is consistent with previous LysK plate lysis data from multiple strains (O'Flaherty *et al.*, 2005), and also resembles the turbidity-reduction sensitivities described for LytA (Donovan *et al.*, 2006b), again consistent with the two enzymes sharing identical peptidoglycan cut sites.

Dependence on C-terminal cell wall-binding sequences for maximal lytic activity has been shown with several peptidoglycan hydrolase enzymes: LytA (Donovan *et al.*, 2006b; Sass & Bierbaum, 2007), lysostaphin (Baba & Schneewind, 1996), ALE-1 (Lu *et al.*, 2006), Ply118, and Ply500 (Loessner *et al.*, 2002). More recently, midprotein Cpl-7 cell wall-binding domains of the streptococcal λSA2 prophage endolysin (Donovan & Foster-Frey, 2008) were shown also to be essential for high activity. However, cell wall-binding domains are not essential for high activity of all peptidoglycan hydrolases for example B30 (Donovan *et al.*, 2006a), PlyGBS (Cheng & Fischetti, 2007), the bacillus

endolysin ply L (Low *et al.*, 2005), and the staphylococcal endolysin plyTW (Loessner *et al.*, 1998). In fact, our results indicating that the LysK CHAP domain requires the SH3b domain for maximal activity are in conflict with the findings of Paul Ross and colleagues who first isolated the *lysK* gene (O'Flaherty *et al.*, 2005). They report that the CHAP domain alone, 165 N-terminal amino acids, has equivalent activity to the full-length LysK protein (Horgan *et al.*, 2009). However, these results may not be contradictory due to the fact that both results arise from non-native constructs, any one of which might alter normal domain function or extinguish regulatory functions inherent to the full-length protein. Additionally, the assay conditions are different between the two labs, 50 mM sodium acetate buffer, pH 6.5 (Horgan *et al.*, 2009), vs. 150 mM NaCl, 10 mM Tris pH 7 (our work). As shown previously, LysK is more active at higher salt, dropping precipitously as the salt concentration approaches zero (Becker *et al.*, 2008). Conversely, when the truncations LysK221 or LysK390 are tested over a salt gradient, higher activities are observed at lower salt concentrations (data not shown) and thus the observed dependence on the SH3b domain may result from differing assay conditions. Alternatively, Low *et al.* (2005) postulated a regulatory model to explain how the absence of the SH3b domain might enhance activity, suggesting that the SH3b domain functions to fold back on and inhibit the lytic domain in the absence of peptidoglycan substrate.

Although the amidase domain appears to be active on purified peptidoglycan in the context of the full-length LysK protein, (consistent with the EIMS results; Fig. 1) and shows limited activity in the absence of the CHAP domain in turbidity-reduction assays and SDS-treated cells in the zymogram (Fig. 2), it is unable to inhibit the growth of cells in either the plate lysis or MIC assays. Interestingly, the staphylococcal phage phi11 endolysin (LytA) amidase domain shares many properties with the LysK amidase domain for example the same peptidoglycan target bond (Navarre *et al.*, 1999), same midprotein location (Wang *et al.*, 1991) and can similarly lyse SDS-treated cells, but is unable to lyse intact cells (heat killed) (Sass & Bierbaum, 2007). Consistent with this minor role in exolysis is the fact that for the majority of staphylococcal strains we tested, deletion of the amidase domain (LysKΔ221-390) did not significantly change the exolytic activity of the protein compared with the wild-type LysK protein. Although the LysK amidase domain is highly conserved among the staphylococcal phage endolysins (D.M. Donovan, unpublished data), our data do not support a role for it in lysis from without, but it may be necessary for its native role in phage particle release from a host cell. It is possible that the amidase domain requires peptidoglycan digestion products that result from CHAP digestion, or cofactors resulting from the phage lytic cycle to achieve maximal activity.

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Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture.

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